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Acetylcholinesterase of *Rhipicephalus (Boophilus) microplus* and *Phlebotomus papatasi*: Gene identification, expression, and biochemical properties of recombinant proteins ☆

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ABSTRACT

The southern cattle tick *Rhipicephalus (Boophilus) microplus* (Bm) is a vector of bovine babesiosis and anaplasmosis. Tick resistance to organophosphate (OP) acaricide involves acetylcholinesterase (AChE) insensitivity to OP and metabolic detoxification. Sequencing and *in vitro* expression of Bm genes encoding AChE allowed biochemical characterization of three BmAChEs expressed in tick synganglion. rBmAChE1, rBmAChE2 and rBmAChE3 exhibited substrate preference for acetylthiocholine, high substrate inhibition and sensitivity to AChE-specific inhibitors. OP-insensitivity mutations were demonstrated in rBmAChE1 and rBmAChE3. Gene silencing suggested functional complementation of BmAChEs *in vivo*. BmAChE genes were amplified in copy number and multiple transcript polymorphisms were expressed in individual tick synganglia for each of the BmAChEs, suggesting allelic diversity within individuals. Studies also identified a gene encoding AChE of the sand fly, *Phlebotomus papatasi*, a vector of leishmaniasis in humans and animals. Expression of recombinant *P. papatasi* AChE (rPpAChE) enabled biochemical characterization and identification of effective inhibitors that selectively target rPpAChE.

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1. Introduction

1.1. Cattle Fever Tick Eradication Program

The southern cattle tick, *Rhipicephalus (Boophilus) microplus*, was discovered to vector bovine babesiosis, also known as cattle tick fever, and anaplasmosis in 1893, which led to an intensive effort initiated in 1907 that culminated in its eradication from the

United States in 1943 [1]. Since 1936, a permanent quarantine zone along the Texas–Mexico border, together with treatment and inspection of all imported cattle has prevented re-establishment of “cattle fever ticks” within the United States. The Texas Animal Health Commission and the Veterinary Services of the Animal and Plant Health Inspection Service of the United States Department of Agriculture manage the Cattle Fever Tick Eradication Program (CFTEP), including inspection and acaricide treatment of

Abbreviations: AChE, acetylcholinesterase; Bm, *Rhipicephalus (Boophilus) microplus*; BmAChE, *Boophilus microplus* acetylcholinesterase; BmAChE1, *Boophilus microplus* acetylcholinesterase 1; BmAChE2, DNA encoding *Boophilus microplus* acetylcholinesterase 2; BmAChE3, DNA encoding *Boophilus microplus* acetylcholinesterase 3; BW284c51, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide; cDNA, complementary deoxyribonucleic acid; CFTEP, Cattle Fever Tick Eradication Program; DNA, deoxyribonucleic acid; dsRNA, double stranded RNA; OP, organophosphate; OP-R, organophosphate resistant; OP-S, organophosphate susceptible; PCR, polymerase chain reaction; PpAChE, *Phlebotomus papatasi* acetylcholinesterase; R86Q, substitution of glutamine for arginine at amino acid position 86; rBmAChE, recombinant *Boophilus microplus* acetylcholinesterase; rPpAChE, recombinant *Phlebotomus papatasi* acetylcholinesterase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RNA, ribonucleic acid; RNAi, RNA interference (gene silencing).

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imported cattle, and eradication of “outbreak infestations” when *R. (B.) microplus* or *Rhipicephalus (Boophilus) annulatus* ticks are discovered within the United States [2].

1.2. Organophosphate targets acetylcholinesterase

A key component of the CFTEP is acaricide treatment of all imported or infested cattle. The only acaricide approved and routinely utilized for dipping cattle in the U.S. since 1970 is the organophosphate (OP), coumaphos [3], and OP-resistance is considered to be a major threat to continued success of the CFTEP [2,4,5]. OP and carbamate pesticides share the mode of action whereby the enzymatic activity of acetylcholinesterase (AChE) in the central nervous system of arthropod pests is inhibited. AChE is a key enzyme in the nervous systems of eukaryotic animals functioning by hydrolyzing the neurotransmitter, acetylcholine. OP targeting of AChE results in quasi-irreversible inactivation of the enzyme, leading to functional collapse of the nervous system and subsequent death of the target species [6,7]. Resistance to OP pesticides has become widespread in arthropod pests throughout the world [8] and is frequently the result of one or more mutations leading to production of an altered AChE that is insensitive to OP inhibition. Molecular structures of AChE from *Torpedo californica*, *Drosophila melanogaster*, and other organisms have been determined by X-ray crystallography and OP target-insensitivity in arthropod pests frequently results from alteration of key amino acid residues located at the catalytic gorge or from “second shell” allosteric effects on the catalytic gorge residues of AChE [9,10].

1.3. Multiple acetylcholinesterase genes in *R. microplus*

Strains of *R. (B.) microplus* that were resistant to OP were biochemically characterized, demonstrating that resistance was due, at least in part, to AChE insensitivity to OP inhibition ([11–13]). Unlike flies containing only a single AChE gene [14], or mosquitoes and some other dipterans that contain two AChE genes [15,16], *R. (B.) microplus* was reported to express at least three different transcripts presumptively encoding AChEs, *BmAChE1* [17], *BmAChE2* [18], and *BmAChE3* [19]. The predicted amino acid sequences of the three encoded BmAChEs were no more closely related to one another than AChEs from different organisms and their specific functional roles remained unknown. In each case, initial surveys comparing cDNA sequences from OP-susceptible or OP-resistant strains failed to identify *BmAChE* mutations that correlated with resistance. In addition, presumptive identification of each of the *BmAChEs*, as encoding AChE, was based solely on nucleotide and amino acid sequence similarity; however, the cholinesterase gene family contains a number of related enzymes and structural proteins [20–23], requiring biochemical verification of the identity of the encoded gene products.

1.4. Baculoviral expression of acetylcholinesterases

The first *R. (B.) microplus* cDNA presumptively encoding AChE to be expressed as a recombinant protein was *BmAChE3*. Biochemical characterization of recombinant BmAChE3 (rBmAChE3) produced in the baculovirus system demonstrated that it was an active enzyme exhibiting biochemical properties consistent with AChE (E.C. 3.1.1.7). It exhibited substrate preference for acetylthiocholine over butyrylthiocholine, was inhibited by the AChE-specific inhibitors eserine and BW284c51, and exhibited similar sensitivity to paraoxon as AChE prepared from OP-susceptible *R. (B.) microplus* adults, thereby verifying the identity as a functional ioxid AChE [24]. Recently, rBmAChE1, rBmAChE2 and rBmAChE3 expressed in the baculovirus system were biochemically characterized [25] and were each found to exhibit properties consistent with AChE

Table 1

Physical and biochemical properties of BmAChE1, BmAChE2 and BmAChE3 of *R. microplus*.

Enzyme ^a	# aa in ORF ^b	Mol. wt. ^b	pI ^b	K _m (AcSch) μM ^c
rBmAChE1	595	65687.6	4.66	5
rBmAChE2	563	61820.3	6.30	50
rBmAChE3	620	69853.9	7.58	90

^a Recombinant BmAChE1, BmAChE2, or AChE3 expressed in baculovirus [25].

^b Calculated from amino acid (aa) sequence in complete open reading frame (ORF).

^c Value of Michaelis–Menten constant for acetylthiocholine (AcSch).

(E.C. 3.1.1.7) as defined for insect AChE by Toutant [26]. Therefore, it is evident that *R. (B.) microplus* has at least three different genes, each encoding an enzymatically active AChE, but each exhibiting substantially different K_m values for acetylthiocholine (Table 1). The present report evaluates genetic and biochemical data for *R. (B.) microplus* AChE gene mutations, providing evidence in support of their role in producing altered BmAChEs with insensitivity to organophosphate inhibition.

1.5. Acetylcholinesterase of the sand fly

Similarly, the sand fly, *Phlebotomus papatasi* (Scopoli), is a major vector for zoonotic and human cutaneous leishmaniasis, affecting millions of people throughout subtropical regions of the world, and a significant problem to U.S. military operations in Iraq and Afghanistan. Control of sandflies is reliant on chemical insecticides, but has had limited success at US military installations in the Middle East [27]. Sand fly resistance to chemical pesticides has been reported [28], but occurrence and mechanisms of resistance remain largely unknown. The cDNA encoding acetylcholinesterase of *P. papatasi* was cloned, sequenced, and expressed in the baculovirus system to generate a recombinant enzyme for biochemical characterization [29]. Recombinant acetylcholinesterases of *Rhipicephalus microplus* and *P. papatasi* were produced and used to screen synthetic carbamates in order to identify novel and efficacious inhibitors [30]. Studies are underway to determine the efficacy of these inhibitors *in vivo*.

2. Methods

Biological material, cloning, sequencing and baculovirus expression of recombinant acetylcholinesterases of *R. microplus* and *P. papatasi* were as described previously [25,29]. Observed mutation frequencies are reported as the number of specific mutations observed divided by the total number of cDNAs sequenced for each BmAChE. We limited observed mutation frequencies to analyses of complete *R. microplus* BmAChE cDNAs cloned from OP-susceptible or OP-resistant strains to provide mutation sampling consistency throughout the coding sequences. Observed mutation frequencies reported are not presumed to represent frequencies *in vivo* due to unequal amplification of different BmAChE genes and presence of multiple alleles for each gene. It is expected that the distribution of specific mutations is not random with respect to individual transcript cDNAs.

3. Results and discussion

3.1. Acetylcholinesterase insensitivity to organophosphate

Association of various *BmAChE* mutations with insensitivity to OP has been evaluated; however, the details of these biological events remain to be fully understood. The R86Q substitution in BmAChE3 was the first mutation shown to confer insensitivity to

OP, resulting in approximately 20-fold reduction in paraoxon sensitivity. The R86Q substitution was presumed to act allosterically because it mapped to the outer shell of the enzyme, well away from the catalytic site [31]. In addition, a recombinant construct of BmAChE1 containing several amino acid substitutions was shown to result in ca. 40-fold reduction in paraoxon sensitivity compared to that of rBmAChE3. Among the 17 amino acid substitutions compared to the wild type BmAChE1 sequence, D188G, E196G, V331A, and F390S were found only in the paraoxon-insensitive construct, and the D188G and F390S substitutions were considered to be most likely candidates responsible for the paraoxon insensitivity [25]. Transcripts expressing the OP-insensitive rBmAChE3 and rBmAChE1 constructs were each isolated from OP-resistant strains of *R. (B.) microplus*, suggesting that phenotypic resistance to OP may involve mutations in more than one of the BmAChEs. Presence of OP-insensitive mutations in susceptible strains further suggests that a single mutation may be insufficient to result in phenotypic resistance to OP as was shown for the R86Q substitution in BmAChE3 [31]. Genotyping surveys of OP-resistant and -susceptible strains utilizing allele-specific assays or cDNA sequencing suggested additional mutations potentially associated with OP-insensitivity ([32]). Based on genotyping studies [25,30–35], sequencing of cDNAs, and biochemical properties of rBmAChEs, a number of amino acid substitutions (encoded by mutations in BmAChEs) were identified that associated with OP-resistant strains or exhibited insensitivity to organophosphate-inhibition in biochemical studies (Table 2). Confirmation of the role of specific mutations in OP-insensitivity requires expression and biochemical characterization of rBmAChEs containing specific mutations individually and in various combinations. Each of the three known BmAChEs is expressed in the adult tick synganglion as determined by qRT-PCR, however, the specific physiological role of each of the individual BmAChEs remains to be fully elucidated. Post-transcriptional gene silencing by RNA interference (RNAi) was utilized to investigate the functional role of the three BmAChEs. Injection of BmAChE dsRNA resulted in specific silencing ($\geq 80\%$) of the target gene, but did not produce significant mortality, unless all three BmAChE genes were silenced simultaneously. Increasing the dose of microinjected dsRNA for individual BmAChEs alone resulted in increased mortality, but remained less than that from silencing all three BmAChEs. These results provide evidence

that the three BmAChEs expressed in the tick synganglion functionally complement one another *in vivo* [33,34].

3.2. Gene amplification of BmAChEs

Efforts to clone and sequence cDNAs from individual ticks that exhibited expression of OP-insensitive AChE activity revealed the presence of multiple different transcripts encoding individual BmAChE1, BmAChE2, and BmAChE3. These results suggested that each of the BmAChE genes may be present in multiple copies, or that they may be subject to alternative splicing [33,34]. Quantitative-PCR utilizing genomic DNA also indicated the presence of multiple copies for each of the BmAChEs in *R. (B.) microplus* [33,34], as did PCR amplification of BmAChE1 genomic DNA fragments encompassing the presence or absence of an intron [35].

3.3. Identification of BmAChE mutations responsible for insensitivity to organophosphate

Analyses of sequence and biochemical data for baculoviral expressed rBmAChEs identified specific amino acid substitutions unique to inhibitor-insensitive constructs of rBmAChE1 [30]. Comparison of amino acid substitutions encoded within 9 cDNAs from OP-susceptible strains with substitutions encoded within 20 cDNAs from OP-resistant strains of *R. microplus* demonstrated that the amino acid substitutions identified in Table 3 as unique to inhibition-insensitive rBmAChE1 constructs were present only in cDNAs from the OP-resistant strains, or in the single OP-susceptible Muñoz #4 cDNA that was demonstrated to encode an inhibition-insensitive rBmAChE1 [30]. This was in contrast to other amino acid substitutions present in the inhibitor-insensitive constructs that were either randomly distributed or most frequently found in OP-susceptible strain cDNAs (Table 3). Similar data are presented for BmAChE2 (Table 4) and BmAChE3 (Table 5); however biochemical studies of expressed recombinant constructs have been much more limited than for BmAChE1.

Of the BmAChE1 amino acid substitutions identified in Table 3 as most likely to be involved in production of insensitivity to inhibition (bold type in Table 3), D188G is located adjacent to tyrosine-187 lining the catalytic gorge and presumed to be a component of the peripheral binding site. F390S is located near tyrosine-388, also presumed to be a component of the peripheral binding site. E196G, V331A, and N566D may exert 2nd shell allosteric effects, and P590A is located near the carboxyl terminal end, potentially influencing membrane attachment or multimeric interactions [9,25,36].

3.4. BmAChEs functionally complement one another *in vivo*

Mutations in BmAChE1 and BmAChE3 have each been characterized as resulting in production of an altered acetylcholinesterase with reduced sensitivity to organophosphate inhibition *in vitro*. In addition, mutations in both BmAChE1 and BmAChE3 have been observed to occur in *R. microplus* strains characterized by bioassay as phenotypically susceptible to OP. It is possible that expression of phenotypic resistance may require multiple mutations within the same gene, or perhaps in multiple genes. The results strongly suggest that investigated strains of *R. (B.) microplus* possess and express multiple copies of each of the three BmAChEs in the tick synganglion that functionally complement one another *in vivo*. Previous studies have demonstrated that the *R. microplus* strain San Román (OP-resistant) produces acetylcholinesterase that is insensitive to paraoxon, exhibits a decreased K_m for acetylthiocholine, and exhibits a metabolic component to resistance [11,12,38]. It may be hypothesized that deleterious effects of BmAChE mutations may be mitigated by gene duplication and maintenance of allelic diversity, including both OP-sensitive and -insensitive

Table 2
Amino acid substitutions^a associated with OP-resistance in *Rhipicephalus (Boophilus) microplus*.

BmAChE1 ^b	BmAChE2 ^c	BmAChE3 ^d
P157S	F9L	I54V
D188G	A26T	R86Q
E195G	E75G	I123M
E196G	W114R	I492M
Y230C	L141F	T548A
S282G	K208R	T548Y
V331A	I210V	
T362A	P343S	
F390S	M349V	
L417P	M406V	
Q488R	K555M	
I493T		
N566D		
W571R		
W571F		
P590A		

^a Amino acid encoded at position number designated is replaced by amino acid following the position number. E.g., P157S indicates the proline (P) at position 157 is replaced by serine (S).

^b Amino acid position indicated in GenBank accession number CAA11702.

^c Amino acid position indicated in GenBank accession number AF067771.

^d Amino acid position indicated in GenBank accession number AY267337.

Table 3Frequencies of encoded amino acid substitutions associated with OP-resistance observed in complete *BmAChE1* cDNAs.

Amino acid substitution encoded in <i>BmAChE1</i> cDNAs ^{a,b}	Observed frequency in <i>BmAChE1</i> cDNAs from OP-susceptible strains (N = 9)	Observed frequency in <i>BmAChE1</i> cDNAs from OP-resistant strains (N = 20)	Observed frequency in <i>BmAChE1</i> cDNAs from all strains (N = 29)
P157S	0.00	0.45	0.31
D188G	0.00	0.20	0.14
E195G	0.11	0.10	0.10
E196G	0.00	0.10	0.07
Y230C	0.00	0.25	0.17
S282G	0.00	0.10	0.07
V331A	0.11	0.30	0.24
T362A	0.00	0.05	0.03
F390S	0.11	0.25	0.21
L417P	0.00	0.15	0.10
Q488R	0.22	0.45	0.24
I493T	0.11	0.05	0.07
N566D	0.00	0.05	0.03
W571R	0.22	0.40	0.34
W571F	0.00	0.10	0.07
P590A	0.00	0.05	

^a Amino acid encoded at position number designated is replaced by amino acid following the position number. E.g., P157S indicates the proline (P) at position 157 (GenBank accession number CAA11702) is replaced by serine (S).

^b Amino acid substitutions listed in bold type were unique to inhibition-insensitive r*BmAChE1* constructs (Table 5 of Ref. [30]).

Table 4Frequencies of encoded amino acid substitutions associated with OP-resistance observed in complete *BmAChE2* cDNAs.

Amino acid substitution encoded in <i>BmAChE2</i> cDNAs	Observed frequency in <i>BmAChE2</i> cDNAs from OP-susceptible strains (N = 11)	Observed frequency in <i>BmAChE2</i> cDNAs from OP-resistant strains (N = 14)	Observed frequency in <i>BmAChE2</i> cDNAs from all strains (N = 25)
F9L	0.09	0.07	0.08
A26T	0.09	0.36	0.24
E75G	0.00	0.14	0.08
W114R	0.00	0.00	0.00
L141F	0.00	0.21	0.12
K208R	0.00	0.00	0.00
I210V	0.00	0.14	0.08
P343S	0.00	0.00	0.00
M349V	0.00	0.14	0.08
M406V	0.00	0.14	0.08
K555M	0.00	0.14	0.08

Table 5Frequencies of encoded amino acid substitutions associated with OP-resistance observed in complete *BmAChE3* cDNAs.

Amino acid substitution encoded in <i>BmAChE3</i> cDNAs	Observed frequency in <i>BmAChE3</i> cDNAs from OP-susceptible strains (N = 14)	Observed frequency in <i>BmAChE3</i> cDNAs from OP-resistant strains (N = 15)	Observed frequency in <i>BmAChE3</i> cDNAs from all strains (N = 29)
I54V	0.86	0.07	0.45
R86Q ^a	0.86	0.07	0.45
I123M	0.00	0.13	0.07
I492M	0.71	0.07	0.38
T548A	0.00	0.00	0.00
T548Y	0.07	0.00	0.03

^a The R86Q substitution was biochemically characterized to result in production of a r*BmAChE3* with reduced sensitivity to paraoxon [31].

alleles [33,37]. It is possible that the presence of multiple copies of *BmAChE1*, *BmAChE2* and *BmAChE3*, expressed in the central nervous system of *R. (B.) microplus*, may provide a significant selective advantage by simultaneous maintenance and expression of multiple alleles and functional complements. In the presence of OP acaricide, expression of OP-insensitive alleles may provide sufficient AChE activity to maintain nervous system function. In the absence of OP acaricide, expression of the OP-susceptible alleles may reduce the fitness cost potentially associated with mutations conferring OP-insensitivity. Maintenance of multiple alleles for multiple *BmAChEs* would also account for previous failures to identify *BmAChE* mutations associated with OP-resistance in *R. (B.) microplus*. It is clear from results of these investigations that AChE participation in *R. (B.) microplus* phenotypic resistance to OP acaricides is complex, involving multiple *BmAChEs*.

3.5. Undiscovered diversity of acetylcholinesterases in ticks

We recently analyzed genomic data from the related tick, *Ixodes scapularis* to produce a phylogram representing amino acid sequences presumptively identified as cholinesterases [37]. In that phylogram, there were at least 9 major branches containing a total of 52 presumptive cholinesterase entries in addition to the three verified *BmAChE* entries. The three biochemically confirmed *BmAChEs* of *R. (B.) microplus* each co-located to different individual branches of the phylogram, strongly suggesting that several additional AChEs remain to be discovered and characterized in *R. microplus* and related ticks.

Acetylcholine and acetylcholinesterase are components of bovine blood. During rapid engorgement, *R. microplus* females may imbibe over 100 times their body weight in host (bovine) blood

within a 5–7 day period [39,40]. The K_m for acetylthiocholine of bovine acetylcholinesterase was reported to be 61 μM [41]. As noted in Table 1, the K_m for acetylthiocholine of BmAChE1 of *R. microplus* is about 5 μM , suggesting that the central nervous system of the tick may have a significantly lower level, and higher sensitivity, for ingested acetylcholine in bovine blood than exists in tick hemolymph. It is reasonable to expect that such large blood volumes would contain significant amounts of acetylcholine that could interfere with normal function of the tick nervous system, suggesting that some tick acetylcholinesterases may function to protect the tick from toxic levels of acetylcholine ingested during blood feeding and engorgement.

Preliminary data (unpublished) suggests that acetylcholinesterase may also be present in tick saliva. Acetylcholinesterases have been reported in saliva of other hematophagous arthropods, including bed bugs [42–44]. Salivary acetylcholinesterase could function in reducing inflammation, modulate the immune response of the host, and promote successful feeding by reducing acetylcholine levels in the host near the attachment site [37,45–49]. In addition, acetylcholinesterases may be critical in reproduction, embryo development and growth as reported for *Tribolium castaneum* [50].

3.6. Recombinant acetylcholinesterase of *P. papatasi*

P. papatasi acetylcholinesterase 1 was found to exhibit very high similarity (92% amino acid identity with *Lutzomyia longipalpis* AChE (partial codes, 396 amino acids, [GenBank: AB174669] and 85% amino acid identity with AChE1 of mosquitoes, *Culex pipiens* [GenBank: Q86GC8], and *Aedes aegypti* [GenBank: XP_001656977]. Recombinant acetylcholinesterase1 of *P. papatasi* expressed and biochemically characterized in baculovirus supernatant exhibited a K_m for acetylthiocholine of $37.9 \pm 1.6 \mu\text{M}$, was sensitive to the acetylcholinesterase inhibitors eserine and Bw284c51, and was insensitive to the butyrylcholinesterase inhibitor iso-OMPA [29].

3.7. Summary – recombinant acetylcholinesterases of *R. microplus* and *P. papatasi*

It is clear from results of these investigations that AChE participation in *R. (B.) microplus* phenotypic resistance to OP acaricides is complex, with evidence strongly indicating the involvement of multiple BmAChEs. As noted in the accompanying paper [30], the biochemical and structural properties of rBmAChE1 appear to be unique among acetylcholinesterases studied to date. In addition, application of the recombinant tick and sand fly acetylcholinesterases to screen synthetic carbamates has identified novel compounds with improved safety profiles relative to mammalian enzymes. Although the specific physiological roles of multiple acetylcholinesterases in hematophagous arthropods remain unknown, accumulating evidence suggests roles in hydrolyzing potentially toxic acetylcholine in the blood meal, modulation of host inflammatory and immune responses, as well as influencing reproduction, development and growth. Further study of the cholinergic system in ticks promises the opportunity to advance discovery and understanding of the complex interactions at the host-parasite interface, as well as to enable the development of novel control applications for sand flies and ticks.

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